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Molecular rearrangement in POR macrodomains as a reason for the blue shift of chlorophyllide fluorescence observed after phototransformation

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Abstract

The activation energy and activation volume of the spectral blue shift subsequent to protochlorophyllide phototransformation (called Shibata shift in intact leaves) were studied in prolamellar body (PLB) and prothylakoid-(PT)-enriched membrane fractions prepared from dark-grown wheat (*Triticum aestivum*, L.) leaves. The measurements were done at 20, 30 and 40 °C and at various pressure values. The activation energy values were 181±8 kJ mol^{-1} and 188±6 kJ mol^{-1} for the PLBs and the PTs, respectively. The pressure stabilized the structure of the NADPH:protochlorophyllide oxidoreductase (POR) macrodomains; it prevented or slowed down the blue shift. There were no significant differences between the activation volumes of PLBs and PTs at 30 or 40 °C giving values around 100–125 ml mol^{-1} which correspond to changes in the tertiary structure of proteins but also resemble the volume changes occurring during the disaggregation of protein dimers or oligomers, or during dissociation of peripheral membrane proteins from membranes. The small differences in the activation parameters of PLBs and PTs indicate that molecular rearrangements inside the POR macrodomains are the primary reasons of the fluorescence blue shift; however, their lipid microenvironment must be also important in the initialization of the shift.

Keywords: Protochlorophyllide; Shibata shift; Prolamellar body; Prothylakoid; Activation volume; Activation energy

1. Introduction

Dark-germinated angiosperms cannot form chloroplasts with photosynthetically active thylakoids; instead etioplasts are developed. Light is not needed for the biosynthesis of the major lipids [1,2], thus a special, lipid-rich inner membrane system – called prolamellar body (PLB) – is formed in the etioplasts. The PLB has hexagonal, paracrystalline arrangement, and the lipids of the tubular network are in cubic phase [3,4]. Planar lamellae, the prothylakoids (PT) are radiating from the PLBs into the stroma of the etioplasts. The MGDG/DGDG ratio is slightly higher in the PLBs than in the PTs [5,6]. MGDG has a tendency to form inverted hexagonal and cubic phase in water, while DGDG forms normal lamellar bilayers [7–9].

There are remarkable differences in the membrane protein compositions of the PLBs and PTs. The main membrane protein of the PLBs is the NADPH:Protochlorophyllide oxidoreductase (POR) enzyme, which accounts for around 95% of its total proteins [6,10–12]. In the PTs other proteins are dominating and POR is present only in minor amounts [6,10–12]. POR is responsible for the light-dependent transformation of protochlorophyllide (Pchlide) into chlorophyllide (Chlide) (for review see [13,14]). The two distinct, photoactive Pchlide complexes with fluorescence emission bands at 644 and 657 nm [15] were ascribed to dimers and oligomers of NADPH:Pchlide:POR ternary complexes, respectively [16,17]. Experiments with chemical cross-linkers [17], fluorescence line narrowing and site selective fluorescence measurements [18] indicate that the

Abbreviations: Pchlide, protochlorophyllide; Chlide, chlorophyllide; PLB, prolamellar body; PT, prothylakoid; POR, NADPH:protochlorophyllide oxidoreductase

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POR subunits are built into macrodomains with regular structure. The small aggregates (dimers) are localized on the surface of the PLBs and have fluorescence emission maximum at 644 nm [15]. The large aggregates are integral components of the PLB membranes and have a fluorescence emission maximum at 657 nm [19,20]. Not all Pchlide molecules are bound to POR, some of them are localized in the PTs and have fluorescence emission maxima at 628 and 633 nm [16,18–20].

Flash irradiation of etiolated plants is often used in studies dealing with Pchlide photoreduction, because the reaction can be initiated in a synchronized way (for review see [21]). After the photoreduction, the esterification of Chlide takes place (for details see [22–26]). As the chlorophyll synthetase activity probably requires monomeric substrate, the highly aggregated POR–Chlide complexes have to undergo conformational changes and/or disaggregation [27]. The disruption of the Chlide aggregates is reflected in a blue shift of the fluorescence emission maximum of the chromophores from 690 nm to 680 nm [27]. This spectral shift – called Shibata shift in case of intact leaves – is completed within 30–60 min after irradiation [28,29]. The molecular background of the process is still not completely clear. In parallel with the Shibata shift, the regular structure of the PLBs is disrupted and vesicles appear. A cubic-lamellar phase transition of the membranes also occurs [30–33]. Several proteins and the lipids of the PLBs are translocated into the developing thylakoids [11,34,35] others are decomposed. After phototransformation, Chlide has to dissociate from POR and bind to chlorophyll synthetase. As fluorescence spectroscopy cannot distinguish between Chl and Chlide molecules in native samples, i.e. protein-bound forms, it does not show if Chlide or Chl molecules or their mixtures are present when Shibata shift is complete. Probably the two processes occur in parallel [24–26].

Several factors such as temperature [36–38], age of the leaves [39,40] and drought stress [41,42] influence the blue shift in vivo. In vitro experiments have shown that the presence of lipids and/or glycerol [43,44], NADPH [33,45,46], different heavy metals [45–47], ATP [48,49] and the pH of the medium [50] have an impact on the macrodomain stability and thus on the kinetics of the shift.

The kinetics of Shibata shift has been analyzed in leaf homogenates prepared from etiolated wheat seedlings, which contained both the PLB and the PT membranes and all other cell components [38]. In these experiments, the temperature and pressure dependence of the process was studied and the activation energy and activation volume of the spectral blue shift were determined. The results indicated that the Shibata shift involves major molecular rearrangements in which the loosened lipid structures play an important role [38]. In leaf homogenates the etioplast inner membranes are mixed with many cell components, which could interact either with the POR complexes or with molecules forming the direct environment of these complexes. These interactions can modify the kinetic properties of the Shibata shift. To avoid these non-specific interactions and to see the effect of the different membrane compositions of the PLBs and PTs on the Shibata shift isolated and purified PLB and PT membranes were studied, i.e. their activation parameters were determined with high pressure fluorescence spectroscopy. The application of different spectroscopic methods at high pressures provides information about protein structure and folding [51], protein disaggregation [52], and different lipid phase transitions [53,54]. It enables also the determination of volumetric aspects of molecular processes [55].

2. Materials and methods

PLBs and the PT-enriched fractions were isolated from 7.5-day-old dark-grown wheat (*Triticum aestivum*, L.) seedlings according to [56]. Glycerol (Sigma) was added to the preparations to a final concentration of 50% (v/v). NADPH was avoided during isolation procedure because it inhibits the Shibata shift [33]. The sucrose concentrations of the PLB and PT preparations were 17.5% and 25% (m/v), respectively. The preparations were characterized at these concentration values and showed great stability. A special experiment was done to study if this concentration difference influenced the blue shift of fluorescence at 20 °C and atmospheric pressure. The sugar concentration of the PLB preparation was increased to 25% and the kinetical parameters of this preparation were compared to those of the preparation with 17.5% sucrose. The difference was smaller than the experimental error in three parallels. (The dilution of PT samples was not done because the PT membranes are stable only in medium with 25% sucrose concentration.) All manipulations were done in dim green light at 5 °C.

Fluorescence emission spectra were recorded at 77 K using a Fluoromax-2 (Jobin Yvon-Horiba, France) spectrofluorometer. To characterize the spectral and photochemical properties of the preparations, some samples were frozen in liquid nitrogen in the dark; others were irradiated with a Chinon 900 C photoflash apparatus (160 J/0.002 s) and were frozen in liquid nitrogen with a 10-s delay. Aliquots of the irradiated samples were dark incubated at 20 °C for 40 min and were then frozen to 77 K.

The temperature dependence of the Shibata shift was studied by recording fluorescence emission spectra at 20, 30 and 40 °C with a FS900CD luminometer (Edinburgh Analytical Instruments, UK). Approximately 200 μl of the sample was pipetted into a cylindrical glass cuvette and was then sealed with a movable teflon piston wearing a rubber O-ring. The cuvette was positioned in the thermostated high-pressure optical cell (Unipress, Warsaw, Poland) in the dark. The optical cell is equipped with sapphire windows that withstand pressure and enable fluorescence measurements. Temperature was controlled with a thermostated jacket. The pressure dependence of the Shibata shift was studied at 40 °C in the pressure range of 0.1 and 100 MPa, and at 30 °C between 0.1 and 50 MPa as in [38]. The pressure was generated by a manually driven pump and measured by a Bourdon gauge (Nova Swiss, Switzerland) with a precision of 1%. (The pressurizing rate was approximately 100 MPa/min and thus the temperature rise was not greater than 1 °C during this manipulation.) The samples equilibrated for 1 min after setting the pressure values. Irradiation was achieved using the excitation beam of the luminometer at 440 nm for 30 s. The bandwidth of the excitation slit was 18 nm, the intensity of the irradiation was 6 $\mu mol\ s^{-1}\ m^{-2}$. Pchlide phototransformation was checked by recording the fluorescence intensity change at 690 nm during the 30-s illumination period [38,57]. To study the spectral blue shift, a series of spectra was recorded at different time intervals after phototransformation. The excitation monochromator was set at 440 nm, the integration time was 0.5 s. The excitation and emission slits were 9.0 and 3.6 nm, respectively.

The spectra were corrected for the wavelength-dependent sensitivity variation of the photomultiplier. In case of spectra recorded at 77 K, 3-point and 5-point linear smoothing was used. This procedure was tested and did not change the structure of the spectra. Three spectra were measured and averaged in this case. Computational analysis was done with the software SPSERV V. 3.14 (copyright Cs. Bagyinka, Institute of Biophysics and Biology Research Center of the Hungarian Academy of Science, Szeged, Hungary) with procedures described in [16].

In order to describe and quantify the kinetics of the blue shift, the exact positions of the emission maxima of the experimental spectra were determined using a Savitzky-Golay fit routine [58,59].

3. Results

Fluorescence emission spectra were recorded at 77 K in order to test the spectral properties of the isolated PLBs and the PT-enriched fractions. The fluorescence spectra of both samples contained the four typical Pchlide forms characteristic for etiolated leaves [16], however, the ratios of the forms were different (Fig. 1A). The main fluorescence band of the isolated PLBs was at 657 nm, only a band of low intensity was present at ~633 nm. The fluorescence bands corresponding to the short wavelength forms became more pronounced in the PTs and the maximum of the spectra was at 654.5 nm (Fig. 1A).

Flash irradiation resulted in phototransformation of the photoactive Pchlide forms in both preparations, the band at ~655 nm disappeared and new fluorescence bands corresponding to Chlide forms appeared at 692.5 and 691 nm in case of the PLBs and PTs, respectively (Fig. 1B). In case of the PT-enriched fractions, a shoulder at ~677 nm could be observed. The position of the band at 633 nm remained almost unchanged. However, the relative amplitude of the short wavelength form increased due to changes in the energy migration conditions and to differences in the fluorescence yield of the Pchlide and Chlide aggregates (Fig. 1A, B). The observed spectral characteristics are in good agreement with earlier data on these preparations [19].

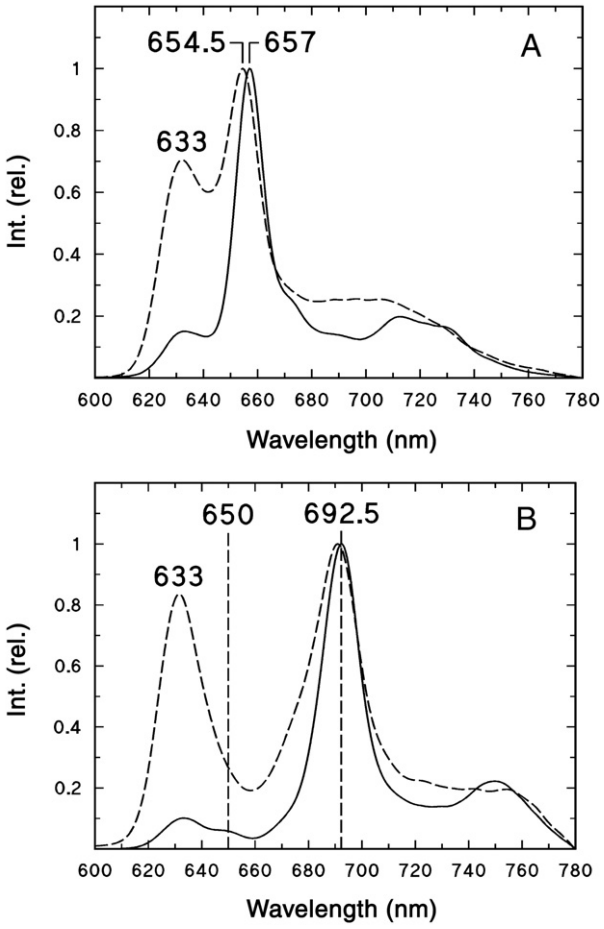

Fig. 1. Fluorescence emission spectra of prolamellar bodies (straight lines) and prothylakoid-enriched fractions (broken lines) isolated from etiolated wheat leaves and recorded at 77 K. All spectra are normalized at their maxima; Int. (rel.) indicates relative fluorescence intensity. A: before irradiation, B: after flash irradiation (160 J/0.002 s).

In further experiments, the spectral changes occurring after irradiation were recorded at various temperatures and pressures. First, the temperature dependence of the process was studied at ambient pressure (0.1 MPa). The fluorescence emission maximum of the first spectrum recorded 61 s after photo-transformation at 20 °C was at 688 nm. The fluorescence bands corresponding to photoactive Pchlide molecules disappeared; only the bands representing non-photoactive Pchlide forms were found in the spectra at around 638 nm (data not shown). This phenomenon was characteristic for all irradiated samples and indicated that the illumination resulted in complete phototransformation. At 20 °C, the blue shift of the emission maximum was practically stopped, but it proceeded very slowly at 30 °C. In order to visualize the progression of the blue shift, the spectra of irradiated PLBs recorded at 30 °C and 0.1 MPa are presented (Fig. 2). In the lowest curve (spectrum recorded 69 s after the beginning of irradiation) the main band corresponding to the newly appeared Chlide form was at 687.1 nm. The blue shift of the emission maximum was relatively slow in this sample; the main band was centered at 682 nm even 115 min after the beginning of the irradiation (Fig. 2, top curve). At 40 °C, the blue shift was relatively fast and was completed within 10–15 min.

In order to quantify the progression of the shift, the maximum position of each spectrum was determined and plotted in the function of time after phototransformation (Fig. 3A). Similarly, the temperature dependence of the blue shift was analyzed in PT-enriched fractions at 20, 30 and 40 °C and 0.1 MPa (Fig. 3B). The starting point of the shift was in this case at 687 nm, while that in the isolated PLBs was at 688 nm. At 40 °C the process was very fast in the PTs: the maximum of the first spectrum was at 683 nm 97 s after illumination (Fig. 3B, squares). At 20 and 30 °C the blue shift was slow and was not completed during the measurements.

At 40 °C, 45 min after irradiation, the emission maxima were at 680 nm and 679 nm in case of the PLBs and PTs, respectively (Fig. 3, squares). These data were used to fit the kinetics in measurements when the end point could not be reached due to the very slow blue shift. The kinetics of the shift was similar for the PLBs and PTs. Based on the data in Fig. 3 a kinetic analysis was performed to determine the rate constants in function of temperature. Assuming first order kinetics, the curves were fitted with single exponentials. The saturation point of the spectral shift was not reached during the measurements at low temperatures (20, 30 °C), in this case the rate constant (k) was determined from the slope of the initial linear part of the exponential decay, which corresponds to $-\Delta\lambda/k$, where $\Delta\lambda$ is the total wavelength shift. From the temperature dependence of the kinetic constants (k) the activation energy was calculated on the basis of the slope of the Arrhenius plot ($\Delta E^{\#} = -R\,\partial\{\ln(k)\}/\partial T$), where T is the temperature (in K) (Fig. 4). This analysis showed practically no difference between the two samples in respect of their kinetic constants, and thus the slope of the Arrhenius plot was very similar for the PLBs and the PTs (for

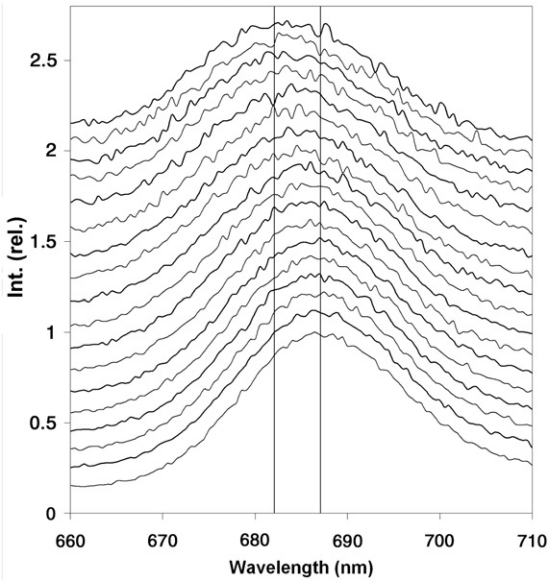

Fig. 2. Fluorescence emission spectra of isolated prolamellar bodies recorded successively after irradiation (6 μmol s^{-1} m^{-2}). Temperature: 30 °C, pressure: 0.1 MPa, excitation wavelength: 440 nm. The vertical lines represent 682 and 687.1 nm. The spectra are shifted along the *y* axis and were normalized at their maxima. The scan of the first spectrum started at 69 s after the onset of the illumination, the consecutive spectra correspond to an illumination period of 226, 387, 547, 705, 900, 1200, 1500, 1800, 2100, 2700, 3000, 3302, 3608, 4027, 4403, 4860, 5120 and 6900 s (top curve).

details and the equations see Fig. 4 and Table 1A). The calculated activation energy values were 181±8 kJ mol^{-1} and 188±6 kJ mol^{-1} for the PLBs and the PTs, respectively.

In order to study the pressure dependence of the blue shift in isolated PLBs and PTs, fluorescence emission spectra were recorded in function of time at 30 and 40 °C and at pressure values between 0.1 and 100 MPa. Pressure delayed the blue shift in case of the homogenates [38], therefore 30 and 40 °C were chosen for the analysis, because at 20 °C the blue shift was already very slow at ambient pressure (Fig. 3, diamonds). At 40 °C, the blue shift was remarkably slowed down at 100 MPa in both preparations (Fig. 5A and B, diamonds).

For comparison, the fluorescence emission maxima of spectra recorded at 0.1 and 30 MPa were also plotted in function of time after irradiation (Fig. 5A and B, squares and circles, respectively). The effect of pressure was similar in the PLBs and the PTs; however, in the PTs pressure had a stronger inhibitory effect on the blue shift at 30 MPa (compare the circles in Fig. 5A and B).

The starting point of the process (obtained after extrapolation) is slightly shifted to the blue with increasing pressure values that is a well-known phenomenon for chromophores [60]. In case of the PTs, the differences in the initial point of the shift are more pronounced indicating the stabilization of the membrane structure and the Chlide form with emission maximum at 690 nm by the pressure (Fig. 5B). At 100 MPa, the saturation point of the blue shift was not reached during the measurements and the fluorescence emission maximum was at 686.5 nm and 685 nm even 120 min after irradiation in case of the PLBs and PTs, respectively.

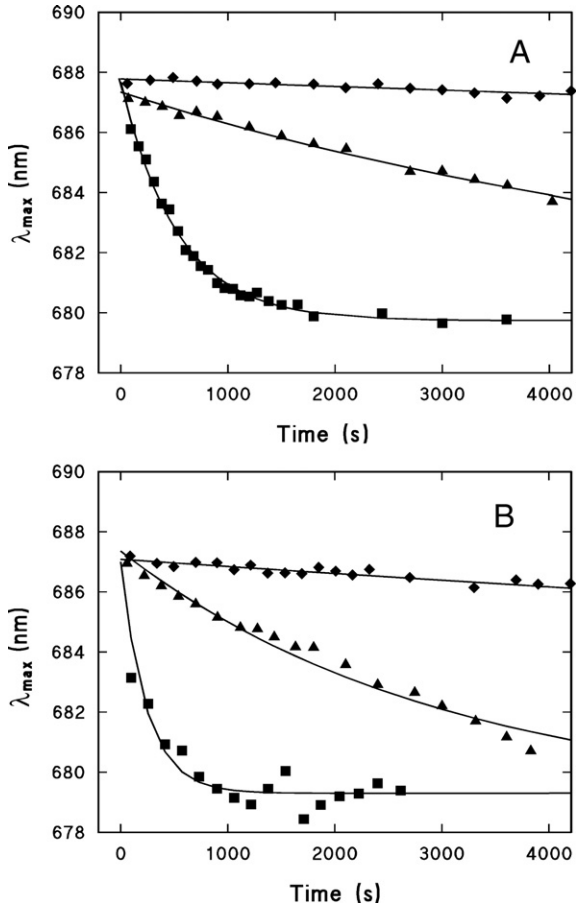

Fig. 3. The positions of the fluorescence emission maxima in function of time after phototransformation at ambient pressure (0.1 MPa) and at different temperatures. Temperature values: 20 °C (diamonds), 30 °C (triangles) and 40 °C (squares). A: isolated prolamellar bodies B: prothylakoid-enriched fractions.

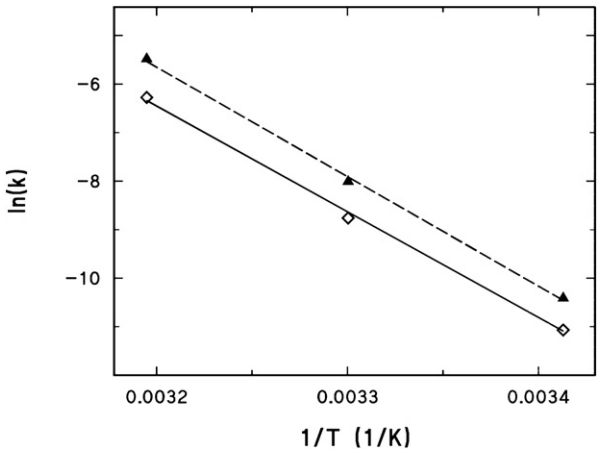

Fig. 4. Arrhenius plot of the reaction rate constant (*k*) of the blue shift in function of 1/T in a semilogarithmic representation at ambient pressure. The kinetic constants were calculated from the monoexponential fit of the blue shift of the fluorescence emission maximum in prolamellar bodies (diamonds) and prothylakoid-enriched fractions (triangles). The data points were fitted with a regression equation corresponding to $\ln(k) = -21800\ K + 63.3$, $r = 0.9989$ in case of isolated prolamellar bodies (straight line) and to $\ln(k) = -22580\ K + 66.6$, $r = 0.9994$ in the prothylakoid enriched fractions (broken line). The activation energy values were calculated from the slopes of the fitted lines.

Table 1
Activation parameters and rate constants of the blue shift of the fluorescence emission maximum after irradiation (corresponding to the Shibata shift in intact leaves) measured in isolated prolamellar bodies (PLBs) and prothylakoid-enriched fractions (PTs)

		PLBs	PTs
(A)			
$\Delta E^{\#}$ (kJ mol^{-1})		181±8	188±6
$k_{40\ °C}$ (s^{-1})		$1.76*10^{-3}$	$3.95*10^{-3}$
(B)			
$\Delta V^{\#}$ (ml mol^{-1})	30 °C	123±36	113±13
$\Delta V^{\#}$ (ml mol^{-1})	40 °C	103±6	116±34
k_0 (s^{-1})	30 °C	$1.33*10^{-4}$	$2.79*10^{-4}$
k_0 (s^{-1})	40 °C	$1.95*10^{-3}$	$1.87*10^{-3}$

(A) Activation energy ($\Delta E^{\#}$) and rate constant values extrapolated at 40 °C ($k_{40\ °C}$). The ln(k) values were calculated from data in Fig. 3. and the activation energy was determined from the Arrhenius plot of Fig. 4. (B) Activation volume ($\Delta V^{\#}$) and rate constant values at atmospheric pressure (k_0) at 30 and 40 °C. The ln(k) values were calculated from data in Fig. 5 and the activation volume was calculated from the plot and equations indicated in Fig. 6.

Similar analysis was done at 30 °C (Fig. 5C and D). In the PLBs, the kinetics of the blue shift was very slow both at 30 and at 50 MPa (compare the similar ln(k) values in Fig. 6), while in the PTs it was only inhibited at 50 MPa. Below these values (30 MPa in case of the PLBs and 50 MPa in the PTs) the blue shift proceeded but was pressure dependent.

The kinetic constants (k) of the spectral blue shift of Chlide were calculated at different pressure values at 30 and 40 °C (Fig. 6, Table 1B). The pressure dependence of the kinetic constants is shown in Fig. 6. As in case of temperature dependence (Fig. 4), the original ln(k) values are indicated in the figure, because this presentation allows the comparison of the kinetics without normalization. The kinetic constants were used to determine activation volumes assuming first order kinetics and using standard thermodynamic treatment, namely $\Delta V^{\#} = -RT\ \partial\{\ln(k)\}/\partial p$ where k is the reaction rate constant, T the temperature (in K), and p the pressure. The activation volume of the spectral shift was determined with linear regression calculation between ln(k) values in function of pressure for both samples (for details and equations see Fig. 6 and Table 1B). The kinetic constants were slightly different in the PLBs and the PTs (Fig. 6, Table 1B). The calculated activation volume values were, however, very similar at both temperatures (Table 1B). At 40 °C, the kinetic constants of the PTs showed large data scattering (Fig. 6, filled triangles). It is probably due to destabilization of the membranes,

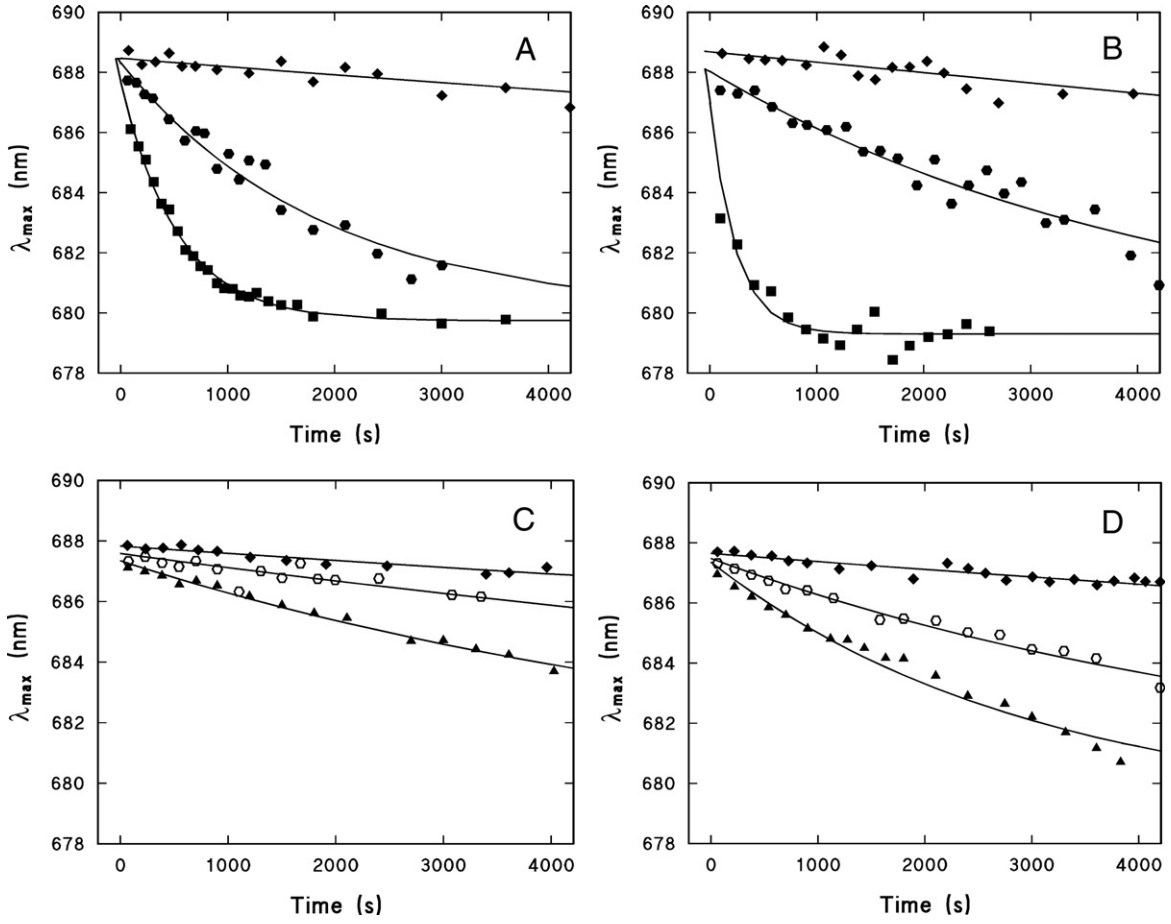

Fig. 5. The positions of the fluorescence emission maxima of isolated prolamellar bodies (A, C) and prothylakoid enriched fractions (B, D) in function of time after phototransformation at different pressures at 40 °C (A, B) and at 30 °C (C, D). A, B: temperature: 40 °C, pressure values: 0.1 MPa (squares), 30 MPa (circles) and 100 MPa (diamonds). C, D: temperature: 30 °C, pressure values 0.1 MPa (triangles), 10 MPa (open circles) and 50 MPa (diamonds).

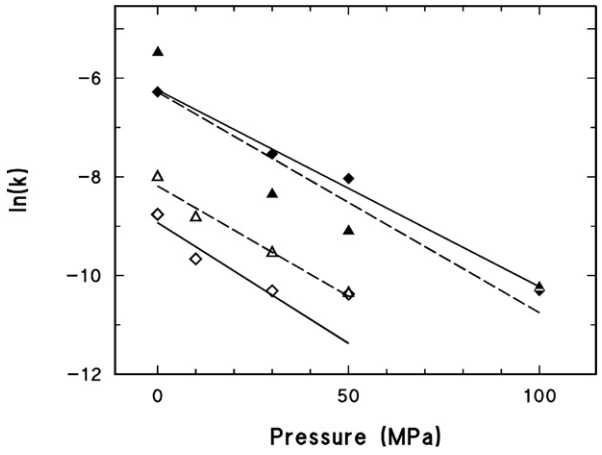

Fig. 6. The reaction rate constant (k) in function of pressure in a semilogarithmic representation at 40 °C (filled symbols) and 30 °C (open symbols). The kinetics of the blue shift was followed in isolated prolamellar bodies (diamonds) and in prothylakoid-enriched fractions (triangles). 40 °C: the data points were fitted with a regression equation corresponding to $\ln(k) = -44.69\ \mathrm{GPa}^{-1}\cdot p - 6.28$, $r = 0.9258$ in case of prothylakoid-enriched fractions (broken line) and to $\ln(k) = -39.88\ \mathrm{GPa}^{-1}\cdot p - 6.24$, $r = 0.9967$ in the isolated prolamellar bodies (straight line). 30 °C: The data points were fitted with a regression equation corresponding to $\ln(k) = -44.73\ \mathrm{GPa}^{-1}\cdot p - 8.18$, $r = 0.9878$ in case of prothylakoid-enriched fractions (broken line) and to $\ln(k) = -48.94\ \mathrm{GPa}^{-1}\cdot p - 8.93$, $r = 0.9601$ in the isolated prolamellar bodies (straight line). The activation volumes were calculated from the slopes of the fitted lines.

therefore, the error of the fit was relatively big and the calculated activation volume should be considered only as an estimation.

4. Discussion

The exact three-dimensional structure of the PLB membranes and the functioning of their main membrane protein, POR, are still not completely understood. The aggregated, photoactive POR–Pchlide–NADPH complexes [15,20] undergo conformational changes and disaggregation after irradiation [17,27,61]. These changes can therefore be studied with fluorescence spectroscopy. The first observable change during Shibata shift is the rearrangement of units within the POR macrodomains that is followed by disaggregation of the Chlide molecules [17,62]. This probably runs in parallel with disaggregation of the POR subunits and with the cubic-lamellar phase transition of the membranes. Directly or indirectly, the structural changes of the membrane lipids play an important role in the blue shift by influencing the conformational changes of POR [38]. The different lipid and protein composition of the PLB and the PT membranes can account for the difference in phase properties and organization [5,6,9]. However, the Pchlide form with emission maximum at 655 nm was also present in the PT fraction. Very small amounts of this form are normally present in the PTs, and thus we cannot exclude the possibility that fragments of PLB membranes, broken during the fractionation procedure, are mixed in this fraction [12].

Temperature and pressure can induce phase transitions of lipids [53,54], thus the fluorescence of Chlide at different conditions can provide information about the molecular background of the Shibata shift and the effects of the different lipid environments on this process. The changes of the fluorescence emission maximum of Chlide could be fitted by one exponential function at all pressure and temperature values in both samples, suggesting that this process has first order kinetics.

At ambient pressure, the blue shift was slow at 20 °C; this can be due to the presence of glycerol and sucrose [52,63]. The experiments were done on PLB preparation with 17.5% sucrose concentration; a higher sugar concentration (in the presence of 50% glycerol) would result in a slow aggregation of the POR macrodomains [64], which would cause difficulties in the interpretation of the results. The blue shift, however, proceeded and speeded up with increasing temperature (Fig. 3). The kinetics of the Shibata shift depends on temperature in intact leaves [36,37] and is strongly affected by the viscosity of the medium described in studies carried out on in vitro preparations [43,44]. The calculated activation energy values (188 ± 6 kJ mol^{-1} and 181 ± 8 kJ mol^{-1} for the PTs and the PLBs, respectively) (Fig. 4, Table 1) indicated strong molecular interactions in the original structures. Disaggregation [17,27], dephosphorylation [48,49] or conformational changes of POR [61], dissociation of Chlide from the enzyme complexes and/or esterification [22,24–26] are processes, which all may proceed during the blue shift. The obtained activation energy is approximately twice of that observed in etiolated wheat leaf homogenates [38]. This can be explained by the fact that the homogenates contained a mixture of all membrane fragments and all kinds of soluble components of the cells, which could have influenced the actual values of the activation parameter. However, it might be possible that the PTs connected to the PLBs influenced the kinetics of the shift and facilitated the disaggregation process within the homogenates.

The pressure dependence of the process was analyzed at 30 and 40 °C, because it was earlier shown that pressure had an inhibitory effect on the blue shift at lower temperatures and these temperature values are critical in the phase transition processes of membrane lipids [38]. The stability of the etioplast inner membranes is low at high temperatures; however, the endothermic transitions observed in the membranes disappeared after irradiation and after the completion of the blue shift [65]. Pressure stabilized the Chlide–POR macrodomain structure within the PLBs and the PTs, which was indicated by the very slow blue shift of the emission maximum at high pressures (Fig. 5). In previous studies on wheat leaf homogenates two components of the blue shift were distinguished [38]. The activation volume of the pressure-dependent component fell within the range of changes proceeding in the tertiary structures of proteins, which can be influenced directly or indirectly by the structural changes of lipid membranes at elevated temperatures [38]. No significant differences were found in the activation volume values of the two membrane preparations at both temperatures (Table 1). This indicates that the measured molecular processes can be linked to changes of the Chlide complexes with emission maximum around 690 nm, which are present in both membranes (however, only in minor amounts in the PTs). The obtained activation volumes correspond to

approximately 172–194 Å^3 volume change per molecule at 40 °C and to 205–189 Å^3 at 30 °C in the PLBs and the PTs, respectively. During the Shibata shift, and thus also the disruption of the pigment aggregates, the disruption of the exciton interaction occurs [62]. The volume changes needed for the initiation of the blue shift can be interpreted on the molecular level by considering the size of the porphyrin ring. This way the volume change could increase the distance of two parallel porphyrin rings by 3.8–4.2 Å and 4.1–4.5 Å at 40 and 30 °C, respectively, which might result in the disruption of direct exciton interaction [66].

The activation volume value of the blue shift falls within the range of changes of tertiary and quaternary structures of the proteins [67]. Dissociation of protein dimers occurs at 120–200 MPa and the volume change linked to this process is approximately 100 ml mol^{-1} [67]. The activation volume of protein–protein interactions is also similar (50–200 ml mol^{-1}) [68,69]. The volume changes accompanying the dissociation of peripheric membrane proteins are 100–150 ml mol^{-1} [67]. This latter process is influenced by the lipid composition of the membrane, which might be reflected in the small difference found between the activation volume and ln(k) values of the PLBs and the PTs. Recently it has been shown that dissociation of POR from the membranes occurs during the light induced transformation of the PLBs [70]. However, the viscosity of the medium influences the actual value of the activation parameters, thus the parameters might differ from those found in other samples [38] or those in vivo. In wheat leaf homogenates the activation volumes were 35 ml mol^{-1} and 39 ml mol^{-1} at 40 and 30 °C, respectively [38] indicating that the structural changes during activation involve smaller volume changes in the homogenates, probably due to their loosened structure or the presence of other cell components in this sample.

The different lipid environment and lipid phase of the PLBs and PTs [5,6,9] influenced only the kinetics and not the actual values of the activation parameters of the blue shift under the experimental conditions. At 40 °C, 30 and 50 MPa pressures had a more pronounced effect on the PT membranes than on the PLBs and were more effective in preserving the POR macrodomain structure in this sample (Fig. 5A, B, circles, Fig. 6). As this temperature is close to the phase transition temperature of the PLBs [65] it is not surprising that only high pressure values (100 MPa) preserved the macrodomain structure by stabilizing the PLB membranes. At 30 °C, the kinetics of the blue shift was different in the samples. In the PLBs, 30 MPa pressure inhibited almost completely the blue shift by affecting the membranes, while in the PTs the blue shift proceeded. This shows that the physical state of the membranes, their rigidity, and the different lipid phases are important in the initialization of the blue shift, because they influence the conformational changes of their integral component, the POR macrodomains.

In the present experiments carried out at different temperature and pressure values, the activation parameters of the Chlide blue shift were compared in isolated PLBs and PTs. The comparison showed that similar molecular processes could be accounted for the observed spectral changes in both samples. This suggests that molecular rearrangements of the POR macrodomains are the primary reason of the blue shift. The POR complexes are essential components of the PLB and PT membranes determining their fine structures. Therefore, the changes in the POR macrodomains can lead to changes in the lipid phase and in the lipid environment of the complexes resulting in a major membrane reorganization reflected in a change of the kinetics of the blue shift.

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